EPR SPECTROSCOPIC OBSERVATIONS OF A MANGANESE CENTER ASSOCIATED WITH WATER OXIDATION IN SPINACH CHLOROPLASTS

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1. Introduction

We all depend on photosynthesis as the primary source of molecular oxygen in the atmosphere. Nevertheless, the key steps in O_2 evolution have remained a mystery in comparison with the reactions and cofactors important to carbon dioxide assimilation and electron transport. Recently, however, an enzyme which couples chlorophyll photooxidation to the oxidation of water has been partially characterized [1]. It consists of a 65 kM_r protein containing 2 Mn ions an a 6 kM_r cytochrome subunit [1,2]. Although the specific mechanism by which this enzyme operates is unknown, a general mechanism for O_2 evolution, based largely on the yield and kinetics of O_2 release in response to brief light flashes has gained wide acceptance [3]. In this mechanism, 5 intermediate states (so called S_n states) of an unspecified enzyme are formed via electron transfer to photooxidized reaction-center chlorophyll II. This may involve several intervening electron-transport cofactors. The nature of these intermediates is unknown, although manganese has long been known to play an essential role (reviewed in [4]). The release of protons into the interior of the thylakoid membrane sacs following some light flashes suggests that water is oxidized in sequential steps rather than by a concerted 4 electron/4 proton process [5,6]. Free O_2 yield is maximal on the third flash and every fourth flash thereafter [3]. Flash-induced changes in a 6 line manganese EPR signal in chloroplasts at room temperature have been reported [7]. However, the periodicity with flash number could not be monitored. Although this manganese is photooxidized by photosystem II, its relation to O_2 evolution is not yet clear.

We wish to report the first EPR spectroscopic observations of a binuclear or possibly tetranuclear manganese-containing center in spinach chloroplasts. The effects of repetitive flashes indicates an apparent periodicity of 4, and the effects of electron-transport inhibitors indicates an association with the water oxidation reaction.

2. Materials and methods

Broken spinach chloroplasts, prepared as in [8], were suspended in a medium consisting of 3×10^{-3} M EDTA, 25% SHN buffer (0.4 M sucrose; 0.05 M Hepes (pH 7.5); 0.01 M NaCl), 25 μg ferredoxin/ml, and 1×10^{-3} M $NADP^+$ as electron acceptors, and 50% glycerol. Samples at 2–4 mg chl/ml in Suprasil quartz tubes of inner diameter 3.8 mm were then given N flashes ($N = 0, 1, 2, 3, 4, 5$ and 6) from a Nd/YAG laser at 532 nm, 25 ns pulse width and 75 mJ immediately prior to quench cooling in an isopentane bath at $-140°C$. EPR measurements were performed on samples maintained near 10 K.

3. Results

The EPR spectrum shown in fig.1a illustrates the difference between a sample receiving 1 flash and 0 flashes. A previously unreported signal is observed which exhibits at least 16 resolved hyperfine lines, and possibly as many as 20, having an average separation of 75–90 G and a weakly anisotropic g-factor

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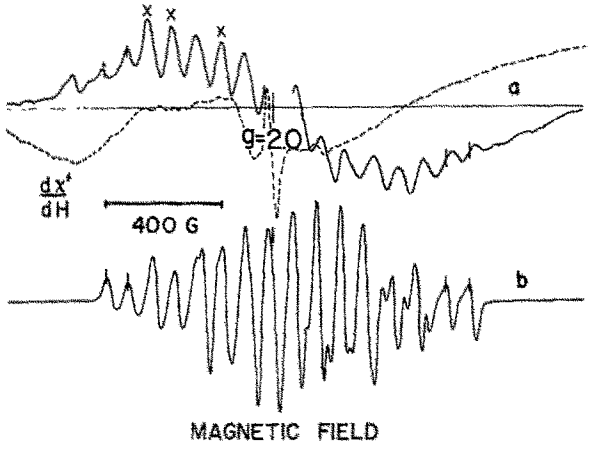

Fig.1. (a) EPR spectrum for spinach chloroplasts in the dark having received 0 laser flashes (———) and the difference spectrum between samples having received 1 and 0 flashes (——) at room temperature followed by quench cooling to −140°C in an isopentane bath. The observation temperature is 10 K; time constants are 1 and 3 s, respectively; microwave power, 150 mW; modulation amplitude, 32 G; microwave frequency, 9.45 GHz; receiver gain, 500. The intensity of the difference spectrum is multiplied by 4 relative to the 0 flash spectrum.

(b) EPR spectrum of $(bipy)_2Mn(III)\begin{smallmatrix}O\\ \diamond\\ O\end{smallmatrix}Mn(IV)(bipy)_2$

Bipy ≡ 2,2'bipyridine; conditions as in [9].

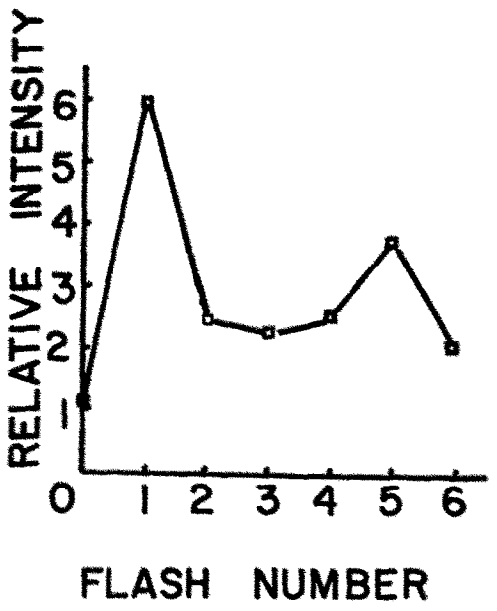

Fig.2. Dependence of the multiline signal of fig.1a on flash number. The average peak height of the 3 largest peaks labelled by × in fig.1 is plotted vs flash number.

of 1.96 ± 0.02 (corrected for 2nd order hyperfine shift). This signal peaks in intensity on the first flash and again on the fifth flash as shown in fig.2. Incubation of chloroplasts with 0.8 M Tris buffer pH 8 and 0.01 M NaCl, which abolishes water oxidation by extracting Mn ions, removes the flash-induced signal. Reduction with 0.05 M sodium dithionite also abolishes this signal. Incubation with the electron-transport inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) has no effect on the first flash. These results indicate a location on the oxidizing side of photosystem II. Extraction of thylakoid membranes with cholate, which has been shown to remove a 65 kM_r protein that is functional in the reconstitution of O_2 evolution and which contains 2 Mn ions [1], also removes the multiline EPR signal (not shown).

4. Discussion

The spectral features in fig.1a are similar to those reported for the mixed-valence complex

$(bipy)_2Mn(III)\begin{smallmatrix}O\\ \diamond\\ O\end{smallmatrix}Mn(IV)(bipy)_2$ shown in fig.1b [9].

Weak transitions which are found in the wings of the chloroplast spectrum are not present in the synthetic dimer spectrum. Preliminary analysis indicates that the apparently larger overall width of the chloroplast spectrum could be due to a binuclear Mn(II)Mn(III) center or possibly a tetranuclear Mn(IV) 3 Mn(III) cluster. The near isotropic g-factor is consistent with high-spin electron configurations around the Mn ions. Oxidation states of a multinuclear Mn center are expected to be EPR detectable when the exchanged coupled Mn ions possess an even number of valence electrons. The hyperfine structure may be simulated with the assumption of inequivalent Mn sites [9]. This shows that the Mn ions have integral oxidation states; the valence is not delocalized. The flash dependence indicates that 1 electron is removed from the Mn ions on each of the first 2 flashes. The fourth flash evidently restores the original starting oxidation state.

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